Genetic Diversity and Discrimination of Chimonanthus praecox (L.) Link Germplasm Using ISSR and RAPD Markers

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Abstract. Chimonanthus praecox (wintersweet) is endemic to China. It has been cultivated there for more than 1000 years as a garden, potted, and cut-flower plant. Many cultivars have been developed during its long history of cultivation, and recently many germplasms were collected in Wuhan and Nanjing, China. The identification and genetic relationship of these resources were studied mainly on morphological traits. In the current study, intersimple sequence repeat markers (ISSR) and random amplified polymorphic DNA markers (RAPD) were used for the first time to investigate 72 wintersweet clones from the two regions. Eleven ISSR primers amplified 115 bands, 105 (78.26%) of which were polymorphic. Nineteen RAPD primers amplified 165 bands, 105 (63.63%) of which were polymorphic. Either ISSR or RAPD markers were sufficient to distinguish all the clones surveyed. A Dendrogram based on Jaccard’s similarity coefficients indicated that the distribution pattern of the 72 clones was coherent with their geographical origins. Most of the genetic variation (85.68% with ISSR data; 86.75% with RAPD data) occurred among clones within each region. However, the difference between Wuhan and Nanjing groups is statistically significant (P < 0.001, with ISSR data; ST = 0.143, P < 0.001, with RAPD data). Morphological variation and classification of wintersweet cultivars were also discussed compared with the genetic relationship based on ISSR and RAPD markers. This is the first report of the partitioning of genetic variability within and between different cultivated wintersweet regions, and it provides useful baseline data for optimizing sampling strategies in breeding. These results are important for future genetic improvement, identification, and conservation of Chimonanthus praecox germplasm.

Chimonanthus praecox (L.) Link, wintersweet, belongs to Calycanthaceae. It is a deciduous shrub and blossoms in winter from late November to March. Its flowers are strongly fragrant, with tepals arranged in three layers. Usually, outer tepals are scale-like, and the middle and inner tepals are waxy and translucent. The unique flowering time and long blooming period made it one of the most popular ornamental plants in China. Wintersweets were extensively cultivated as garden, potted, and cut-flower plants. Also, it is a traditional Chinese medicinal plant for the treatment of heatstroke, cough, scald, and bruise (Zhao et al., 1993). In recent years, this plant has been brought further attention as a new source of natural essential oil, which can be used in cosmetics, perfumery, and aromatherapy.

The natural distribution areas of wintersweet are west of the Hubei and Hunan provinces, south of Shanxi province, and east of Sichuan and Guizhou provinces in China (Chen and Chen, 1999). The plant has been cultivated for more than 1000 years as an ornamental plant. It was introduced to Korea between 1611 and 1628 (Feng et al., 1990), then to Japan, Europe, America, and Australia.

A Dendrogram based on Jaccard’s similarity coefficients indicated that the distribution pattern of the 72 clones was coherent with their geographical origins. Most of the genetic variation (85.68% with ISSR data; 86.75% with RAPD data) occurred among clones within each region. However, the difference between Wuhan and Nanjing groups is statistically significant (P < 0.001, with ISSR data; ST = 0.143, P < 0.001, with RAPD data). Morphological variation and classification of wintersweet cultivars were also discussed compared with the genetic relationship based on ISSR and RAPD markers. This is the first report of the partitioning of genetic variability within and between different cultivated wintersweet regions, and it provides useful baseline data for optimizing sampling strategies in breeding. These results are important for future genetic improvement, identification, and conservation of Chimonanthus praecox germplasm.

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Table 1. List of the 72 *Chimonanthus praecox* clones included in the study.

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<thead>
<tr>
<th>Clone</th>
<th>Original name</th>
<th>Origin</th>
<th>Color of inner tepals</th>
<th>Color of medium tepals</th>
<th>Shape of medium tepals</th>
<th>Flower size</th>
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*C1, yellow; C2, yellow sprinkled with a few mauve speckles; C3, yellow sprinkled with mauve blotches; C4, mauve.

*Flower size was described by Chen et al. (2004).*
Inter-simple sequence repeat marker and RAPD amplifications. Inter-simple sequence repeat marker amplification was performed in a 25-μL reaction volume, containing 40 ng of template DNA, 1× PCR buffer (MBI Fermentas, Lithuania), 2 mM MgCl2, 0.2 mM each of dNTP, 0.2 μM ISSR primer (Sangon, Shanghai, China), 2% formamide (Sigma, St. Louis), and 1 unit of Taq DNA polymerase (MBI Fermentas). All PCR reactions were run on a PE 9600 Thermal Cycler (Perkin Elmer Corp., Waltham, MA) using the following reaction condition: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 51 to 53 °C, and 90 s at 72 °C, with a final extension of 7 min at 72 °C. Polymerase chain reaction products were separated on 2% (w/v) agarose gels containing 0.5 μg mL−1 ethidium bromide in 1× TAE buffer, and were photographed under ultraviolet light using a Gel-Logic 200 image system (Eastman-Kodak, Rochester, NY).

Random amplified polymorphic DNA marker amplifications were carried out in a 20-μL reaction volume containing 20 ng of template DNA, 1× PCR buffer, 2.25 mM MgCl2, 0.1 mM each of dNTP, 0.4 μM RAPD primer (Sangon, Shanghai, China), and 1 unit of Taq DNA polymerase. The mixture was subjected to PCR using the following conditions: 1 cycle of 94 °C for 3 min, 36 °C for 1 min, 72 °C for 3 min, and 35 cycles of 94 °C for 50 s, 36 °C for 50 s, and 72 °C for 2 min, followed by a final extension of 72 °C for 7 min. Products were analyzed on 1.4% agarose gels containing 0.5 μg mL−1 ethidium bromide, and photographed.

Data analysis. The ISSR and RAPD bands were scored as presence (1) or absence (0), and only those that were well defined and consistently repeatable in two independent amplifications were included in the analysis. Similarity matrices based on Jaccard’s similarity coefficient was used to construct a UPGMA (unweighted pair group method with arithmetic average) dendrogram. Correlation between the ISSR and RAPD matrices was estimated using the Mantel matrix correspondence test. These analyses were performed using NTSYS-PC version 2.1 (ExeterSoftware, Setauket, NY). Statistical stability of the branches in the dendrogram was estimated by bootstrap analysis with 1000 replicates, using the Free Tree 0.9.1.50 software program (Pavlicek et al., 1999).

To evaluate the discriminatory power of molecular markers, polymorphic information content (PIC) and marker index (MI) were calculated. The PIC value was determined by applying the simplified formula (Roldán-Ruiz et al., 2000) PIC = 2f 1 [1 − f] where f is the percentage of the amplified allele (band present) and 1 [1 − f] is the frequency of the null allele (band absent) of marker i. The MI was determined as a product of PIC and the number of polymorphic bands per assay unit. An analysis of molecular variance (AMOVA) was used to analyze the partition of molecular variation among wintersweet clones. The Euclidean metric distance of Excoffier et al. (1992) was used in the AMOVA. The homogeneity of molecular variance analysis (HOMOVA), based on Bartlett’s statistic, was also applied to test whether there was significant differentiation between two regions. Bartlett’s statistics null distributions were obtained after 1000 permutations. Both AMOVA and HOMOVA were performed using WINAMOVA 1.55 software.

Results

Inter-simple sequence repeat marker analysis. Of 57 ISSR primers tested, 11 showed clear, reproducible band patterns and were chosen for this study. A total of 115 fragments, ranging in size from 280 to 2200 bp, were identified, and 90 of them (78.26%) were polymorphic. Between 5 (UBC844) to 14 (UBC848) fragments were amplified per primer, with an average of 10.45 bands. The number of polymorphic fragments for each primer varied from 3 (UBC844) to 13 (UBC848), with an average of 8.18. Average PIC and MI were 0.22 and 1.91 respectively.

None of any pair accessions exhibited identical band patterns. The amplified ISSR bands were adequate to discriminate all clones. A 530-bp-long band generated by primer UBC841 was a unique band for H35. In six different cases, there was a unique fragment absent.

The polymorphic bands were used to generate the similarity matrix. Jaccard’s similarity coefficient ranged from 0.42 (between H10 and N25) to 0.96 (between H13 and H22), with a mean similarity of 0.60. The similarity coefficients between two pairs of cultivars, H2 and H5 (both were named as ‘Zhaoxiao’, 0.87), and H13 and H68 (both were named as ‘Acuticoncolor’, 0.77) were lower than expected, indicating two cases of homonymy existed.

Random amplified polymorphic DNA marker analysis. Of 180 arbitrary primers screened in this study, 19 were selected because of their ability to produce reproducible and well-defined bands. A total of 165 fragments were generated across the 72 clones, ranging in size from 200 to 2000 bp. Of these, 105 (63.63%) were polymorphic. The number of bands produced by each primer varied from 4 (OPC14) to 14 (OPC15 and OPK17), with an average of 8.68 bands. The polymorphic bands ranged from 2 (OPC4) to 12 (OPC15), with an average of 5.53 bands per primer. Average PIC and MI were 0.18 and 1.08 respectively.

Similar to ISSR analysis, the amplified RAPD bands can discriminate all clones analyzed in this study. Some bands were unique to a single genotype. The 1500-bp-long band produced by the primer OPK9 was uniquely identified in H29, whereas the 1200-bp band of primer OPC14 and the 850-bp band of primer OPC15 were only observed in H46.

Jaccard’s similarity coefficient varied between 0.46 (between H25 and H29) and 0.97 (between H13 and H22), with an average of 0.62. The similarity coefficient between genotype H2 and H5 was 0.85, and between H13 and H68 was 0.82, also indicating the existence of homonymy.

The correlation coefficient between RAPD and ISSR data sets was 0.62, indicating that the two methods had a positive correlation in this study.

Dendrogram obtained with ISSR and RAPD markers. Because the combined data would give a better coverage of the genome (Siragusa et al., 2006), ISSR and RAPD data sets were combined for UPGMA cluster analysis. Eleven clusters were defined (Fig. 1). The distribution pattern of the 72 clones was coherent with their geographical origins. All the clones of Nanjing origin, with the exception of N25, were included in clusters 06 and 07. The other clusters consisted of all the clones from Wuhan plus N25. In the dendrogram, there were only a few subclusters associated with particular morphological characteristics. For instance, four clones with light-green tepals (H33, H37, H38, and HY) were grouped together at similarity of 0.7 in cluster 02. Cluster 09 contained six clones (H11, H64, H13, H22, H68, and H44), all having yellow inner tepals. The other clusters were composed of clones with wide phenotypic range.

Analysis of molecular variance and HOMOVA. To verify the result that wintersweet clones clustered coherent with their geographical origin, AMOVA and HOMOVA were performed. Analysis of molecular variance provides an estimated FST of population differentiation, which is equivalent to an FST statistic when the degree of relatedness among the genetic variants is evaluated (Belaj et al., 2004). The majority of the total genetic diversity (85.68% with ISSR data, 86.75% with RAPD data) was attributed to differences among clones within a geographical region (Table 2). However, a significant F value (FST = 0.143, P < 0.001, with ISSR data; FST = 0.132, P < 0.001, with RAPD data) suggested the existence of differentiation between clones from Wuhan and Nanjing. Corresponding HOMOVA also indicated that the molecular variances were heterogeneous between two regions (B = 0.049, P = 0.039, with ISSR data; B = 0.217, P < 0.001, with RAPD data).

Discussions

Genetic diversity. Wintersweet is a dichogamous, predominately outcrossing species. Therefore, a substantial level of genetic diversity was detected among cultivated germplasm (average ISSR-based and RAPD-based genetic similarity was 0.60 and 0.62 respectively). When AMOVA was used to analyze the partition of molecular variation, both ISSR and RAPD data detected a high genetic differentiation among clones within each region (85.68% with ISSR data, 86.75% with RAPD data). This higher variability within a population agrees well with the allomagous nature of wintersweet. Similar results were also obtained in natural
wintersweet populations (Chen et al., 1999), and cultivar collections of olive (Belaj et al., 2004) and sweetpotato (Zhang et al., 1998).

Nevertheless, significant $\Phi_{ST}$ and HOMOVA values showed divergence to some extent between the clones of Nanjing and Wuhan. The differentiation may be explained by different selection criteria taken by gardeners for different traits. The result indicates that the two regions harbor different germplasm resources for widening the genetic base in future breeding, and a greater effort should be taken at collecting and hybridizing wintersweet cultivars from both regions.

**Discrimination and classification.** Morphological traits, like medium tepal color, inner tepal color, tepal shape, flower shape, and flower size are usually used to identify wintersweet cultivar. Nevertheless, these traits displayed a continuous phenotypic range and were inferred as quantitative traits dominated by multigenes (Chen et al., 1990). In addition, morphological characteristics are considerably affected by environmental factors. Therefore, accurate identification based merely on morphological traits is difficult. For example, H2 and H5 (both were named as ‘Zhaoxia’), H13 and H68 (both were named as ‘Acuticoncolor’), H37 and H38, and H34 and H45 are difficult to separate based on morphological characteristics. However, in this study, using ISSR or RAPD, they can be easily distinguished. The situation reinforces the importance of using molecular markers for precise identification. Moreover, some fragments were uniquely amplified or absent in a single accession. These fragments are of great interest in optimal management and identification of germplasm collections.

In the existing classification of wintersweet cultivars (Chen et al., 2004; Feng et al., 1990; Zhao et al., 1993), special emphasis was put on the inner tepal color, ranging from yellow, yellow sprinkled with a few mauve speckles, yellow sprinkled with mauve blotches, to mauve. However, the current study demonstrated that although all clones in cluster 09 had yellow inner tepals, in general, clones with the same inner tepal color were dispersed into all groups. Based on morphological cluster analysis, Zhao et al. (2004) found a similar result. In a progeny test, Wu (1992) found that 3.6% to 25.0% of the offspring of individuals with yellow inner tepals had inner tepals with more or less mauve blotches, whereas 5.9% to 44.4% of the offspring of individuals with mauve inner tepals had yellow inner tepals. In this study, clones with the same inner tepal color being clustered into different groups in the dendrogram agreed with these previous results, indicating that clones with similar inner tepal color are genetically heterogeneous.

Furthermore, middle tepals of wintersweet showed different colors, from whitish yellow to greenish yellow to yellow to golden. Zhao et al. (1993) proposed the middle tepal color as the first grade of classification criterion. In the current study, clones were not clustered according to flower color.
only greenish yellow flower clones (H33, H37, H38 and HY) could be grouped together in cluster 02. However, in wintersweet, greenish yellow flowers is a very rare characteristic. Only four such clones were collected in this study; their relatively high similarity (0.7) may indicate they were developed from a common ancestor. In the dendrogram, clones with same tepal shapes, flower shapes, or flower sizes were also dispersed in all clusters.

In general, the molecular classifications were less or more follow morphological classification. However, in our study, discrepancy exists between morphological characteristics and molecular data. This might be most likely the result of the primary stage of winter-sweet’s breeding, during which most cultivars and varieties derived from open pollination, artificial selection, and maintained by vegetative propagation. There does not yet exist “breeding pools” intended as germplasm owned by a breeder that is repeated intercrossed within it. In similar studies characterizing olive (Hagidimitriou et al., 2005) and almond cultivars (MirAli and Nabulsi, 2003), very low correlation was found between the molecular data and the morphological data. Based on the result, Hagidimitriou et al. (2005) proposed that morphological traits were not reliable in estimating genetic relationships among large and diverse groups of cultivars.

**Amplification of ISSR and RAPD.** The reliability of RAPD is widely discussed; nevertheless, in our study, PCR conditions were well set up, and only well-defined fragments were included in analyses. Therefore, good reproducibility was achieved with both RAPD and ISSR markers. Many studies have reported that the ISSR technique is highly efficient and reproducible (Arnau et al., 2003; Levi et al., 2004). In this study, higher PIC and MI values were achieved with ISSR than with RAPD, confirming ISSR markers were more informative because of their greater ability to detect polymorphism. Correlation analysis indicated a positive relationship (r = 0.62) between ISSR and RAPD; the r value was lower than the study in sour orange (r = 0.93) (Siragusa et al., 2006), but higher than the study in blackgram (r = 0.32) (Souframanien and Gopalakrishna, 2004). For two independently derived data sets, a Mantel correlation value (r) greater than 0.5 will be statistically significant at the 0.01 P level if the number of observed taxonomic units exceeds 15 (Souframanien and Gopalakrishna, 2004).

Poly (AT) dinucleotide repeats are thought to be the most abundant motifs in plants (Morgantae and Olivier, 1993); nevertheless, ISSR primers based on (AT)n yielded no amplification products in this study. The result is consistent with the findings of Blair et al. (1999) for rice and Moreno et al. (1998) for grapevine. This may indicate that wintersweet genome lacks the (AT)n motif or that it has very few of these microsatellites.

In conclusion, our results indicate ISSR and RAPD markers are useful for distinguishing and characterizing wintersweet germplasm. The genetic relatedness among these genotypes could provide useful information for conservation and selection of cross-parents in breeding.

**Literature Cited**


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